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Activation of Hh Signaling: A Critical Biological Consequence of ETS Gene Anomalies in Prostate Cancer

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14. ABSTRACT

One of the more notable early molecular changes in prostate cells associated with neoplastic development involves the acquisition of genetic anomalies (chromosomal rearrangements or deletions) that increase expression of gene products of the ETS family (exemplified by ERG, ETV-1, ETV-4 or ELK-4). We propose that one important consequence of ETS gene overexpression in prostate cells is increased expression and activity of Gli transcription factors that are normally induced by classical Hedgehog signaling. To this end, we have identified that GLI1 is regulated by androgens in both LNCaP and VCaP prostate cancer cells. We also under the process of generating the ETS-overexpressing benign prostate cells to test the consequences of ETS overexpression on hedgehog signaling. Additionally, we identified Gli overexpression induces androgen-independent growth of prostate cancer cells and this action is mediated by interaction between GLIs and androgen receptor (AR) which leads to activation of AR signaling under androgen-deprived conditions.

15. SUBJECT TERMS
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Annual Progress Report

DOD Project: W81XWH-10-1-0125
Title: *Activation of Hh Signaling: A Critical Biological Consequence of ETS Gene Anomalies in Prostate Cancer*
Principal Investigator: Mengqian Chen, Ph.D.
Progress Period: 1 March 2010 through 28 February 2011

INTRODUCTION: Although prostate cancer is the most frequent malignancy found in men, the cause of this disease remains enigmatic. None the less, we are gaining in our understanding of the genetics of the prostate cancer cell and the early molecular events associated with the acquisition of malignant characteristics. One of the more notable early molecular changes in prostate cells associated with neoplastic development involves the acquisition of genetic anomalies (chromosomal rearrangements or deletions) that affect genes of the ETS family. These anomalies result in increased expression of an ETS gene product (exemplified by ERG, ETV-1, ETV-4 or ELK-4) since the rearrangements bring an ETS gene coding sequence under the control of a gene promoter that is more transcriptionally active in prostate cells compared to the normal ETS gene promoter (1-4). We propose that one important consequence of ETS gene overexpression in prostate cells is increased expression and activity of Gli transcription factors that are normally induced by classical Hedgehog signaling (5-6). This hypothesis has several important implications since overactive Gli shares properties in common with ETS overexpression including an increased motile phenotype and ability to synergize with other genetic changes to induce overt malignancy. The work in this project will test whether ETS genes activate Hedgehog signaling by turning on Gli expression and activities in prostate cells. We will also explore the downstream signaling profile regulated by ETS or GLI overexpression in prostate cells to determine whether molecular events induced by ETS gene anomalies are mediated by GLI activation. Additionally, we will test whether Gli reactivation, induced by ETS family genes, affect androgen signaling in prostate cells and induce androgen-independent growth of prostate cancer cells.

BODY: This project has 3 Specific Aims and progress will be discussed for each Aim.

Specific Aim 1. Determine the relationship between the expression of ETS genes (ERG, ETV1 or ETV4) and expression and activity of Gli (1 and/or 2) transcription factors in benign and malignant prostate cells. (Month 1-12)

Work Done: We first confirmed that GLI1 gene expression is induced by androgen treatment in two prostate cancer cell lines known with ETS gene fusion, LNCaP and VCaP cells (Figure 1). In both cell lines, GLI1 gene expression is upregulated by R1881 treatment in a dose-dependent manner. In VCaP cells, we confirmed the reported ETS-gene fusion, ERG fused with TMPRSS2 gene promoter, and that ERG is upregulated by androgen in a similar dosage-dependent manner. In LNCaP cells, the ETV1 gene is highly expressed but is not very dependent on androgen signaling.

We further tested whether androgen-regulated GLI1 expression in LNCaP cells is dependent on ETV1 overexpression by an siRNA knockdown experiment (Figure 2). Surprisingly,

downregulation of ETV1 expression did not reduce GLI1 expression to any extent in either no-androgen or androgen-treated conditions. Combined with our data that ETV1 was not androgen-regulated in LNCaP, we feel that it is likely that there is some other members of ETS family, regulated by androgen through gene fusion, is mediating androgen-dependent activation of GLI1 in LNCaP cells. Currently we are performing the microarray analysis for gene expression profiles between androgen-treated and untreated LNCaP cells to identify potential ETS candidates that are regulated by androgen in LNCaP cells. We are also now testing the hypothesis that androgen-dependent regulation of GLI1 in VCaP cells is mediated by androgen-stimulated ERG by knockdown ERG expression in VCaP cells with gene-specific siRNAs.

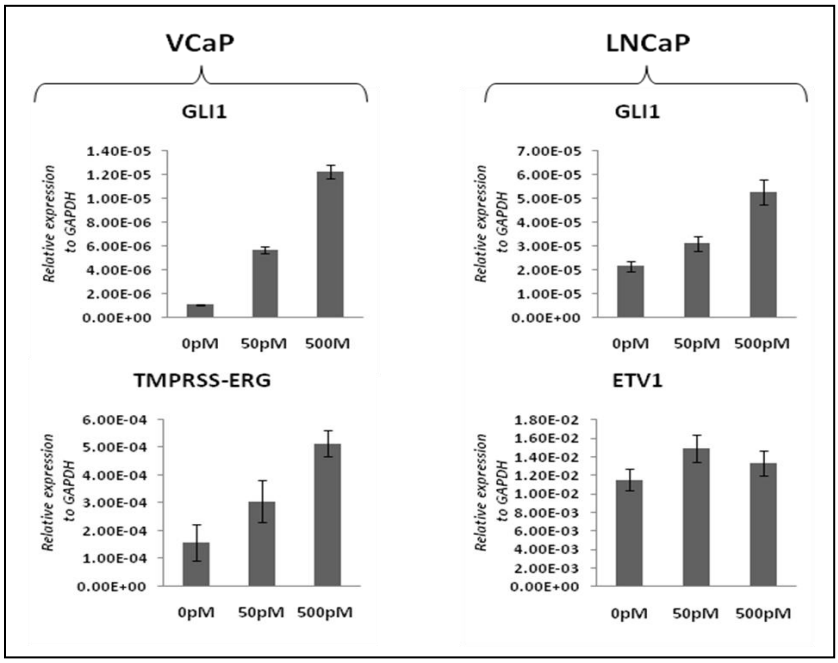

Figure 1. Gene expression of GLI1 and ETS genes in VCaP and LNCaP cells treated with androgen. VCaP and LNCaP cells were seeded into 60mm dishes at density of $5x10^5$ cells per dish and cultured in Charcoal-stripped FBS (CS-FBS) media for 3 days. After androgen deprivation, cells were treated with R1881 at indicated concentration for 24 hours before lysed for RNA extraction and quantitative PCR for relative mRNA expression level of indicated genes. Data are representative of duplicate experiments.

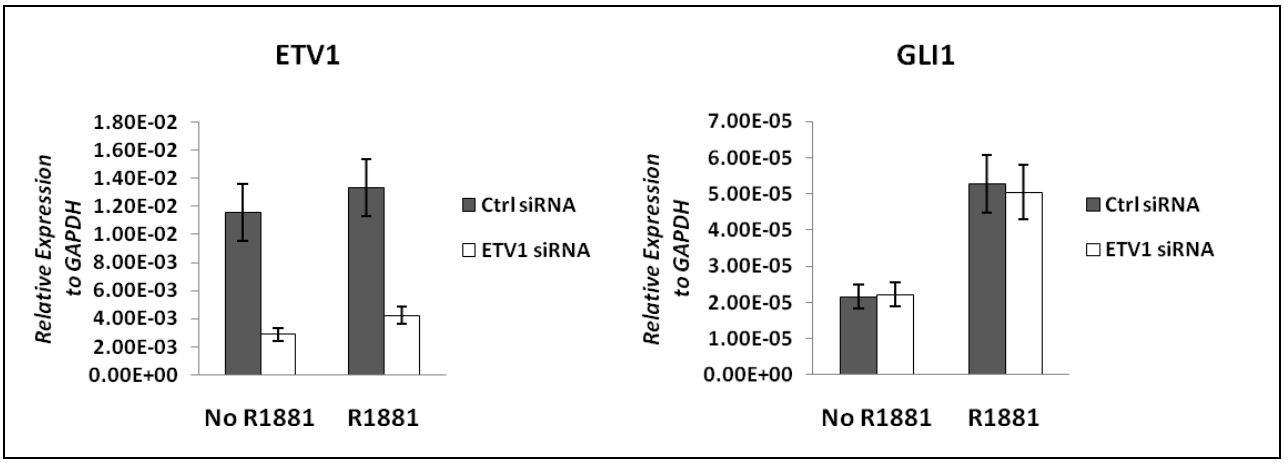

Figure 2. Knock-down ETV1 in LNCaP cells does not affect GLI1 expression in LNCaP cells. LNCaP cells were transfected with 20nM ETV1 siRNA or non-specific Ctrl siRNA for 48 hours and further cultured in CSFBS media for 48 hours. Cells were treated with or without 500pM R1881 for 24 hours before RNA extraction and qPCR analysis for ETV1 and GLI1 mRNA expression. Data are representative of duplicate experiments.

Ongoing Work (To be completed). We also successfully sub-cloned the cDNAs of ERG, ETV1, and ETV4 genes into lentiviral-based expression vectors to generate stable-expression benign prostate cell lines (RWPE1 and BPH1). Currently the transfected cells are under drug selection and we will test whether overexpression of these ETS genes in benign prostate cells turns on GLI expression after we collect the stable expression clones.

Specific Aim 2. Determine whether ERG overexpression, through Gli, induces expression and activity of Polycomb Group proteins (Month 10-20)

At the same time we are waiting for the stable ERG overexpression benign and malignant prostate cells, we already generated Doxycyclin (Dox) -inducible GLI expression LNCaP cells to profile the gene expression pattern regulated by GLI overexpression (Figure 3). We are under preparation of mRNA for mciroarray-based gene profiling using Affymetrix ST1.0 gene array Chips. No other work was done on this specific aim during the last period because the ERG-overexpressing benign prostate cells are not ready yet.

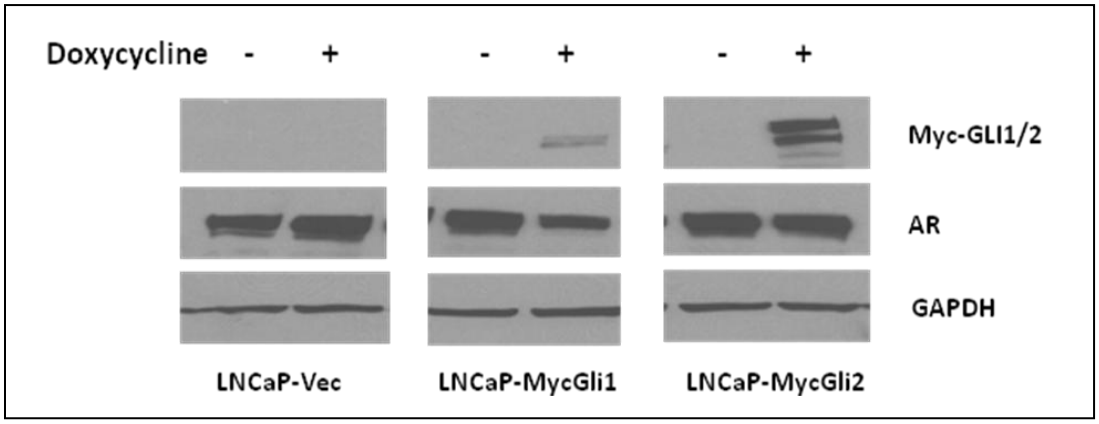

Figure 3. Western blot for Myc-tagged GLI1/2 and AR expression in Dox-inducible GLI expression LNCaP cells. Cells were seeded in regular culture media and treated with or without 100ng/ml Doxycycline for 48 hours. Cells were lysed in RIPA lysis buffer and analyzed for protein expression of Myc-tagged GLIs, AR and GAPDH with specific antibodies.

Specific Aim 3. Determine whether ERG overexpression in benign prostate cells induces gene methylation and gene silencing (Months 12-24).

No work was done on this specific aim during the last period because we are still waiting for the stable ERG overexpression benign prostate cells.

Additional work was also undertaken outside of original Specific Aims. Though not specified in the original Specific Aims, we feel that this work is relevant to the outcome of this project.
GLI transcription factors activate androgen signaling. We identified that all GLI transcription factors (GLI1/2/3) are able to interact with androgen receptor (AR) when they are co-expressed in FT293 cells (Figure 4) and enhance the transcriptional activity of AR on

androgen-responsive promoter reporter under either androgen-free or androgen-supplemented conditions (Figure 5). This novel function of GLIs on AR activity provide a potential mechanism of ETS action on androgen signaling in prostate cancer cells through up-regulation of GLIs. As a fact, we did observe that changes of Hedgehog/GLI signaling in prostate cancer cells affect androgen-regulated genes and androgen-dependent growth in our recent publication (7). All these findings support the idea that activation of Hh/Gli signaling might be a critical consequence of prostate cancer progression mediated by the ETS gene anomalies.

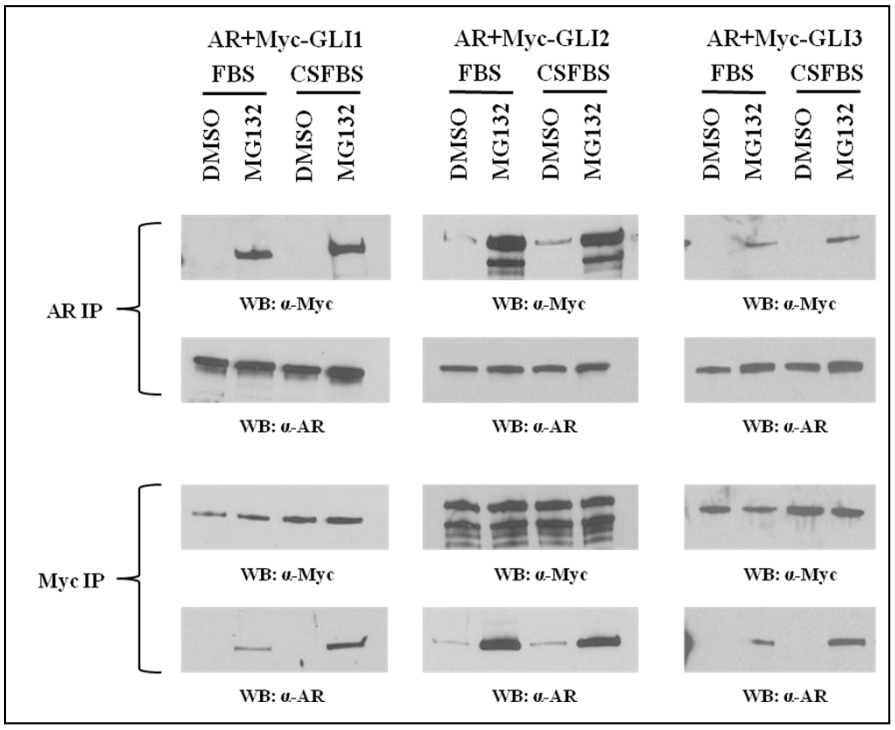

Figure 4. Androgen receptor (AR) interacts with GLIs in FT293 cells. A human AR cDNA expression plasmid was co-transfected with Myc-tagged GLIs (GLI1/2/3) expression plasmid into FT 293 cells under FBS or CSFBS culture condition. 48 hours after transfection, cells were lysed and protein-protein interaction was analyzed by immunoprecipitation (IP) with Anti-AR or Anti-Myc Antibodies. AR-GLI interaction was greatly enhanced by MG132 treatment (20μM) for 4 hours before protein extraction.

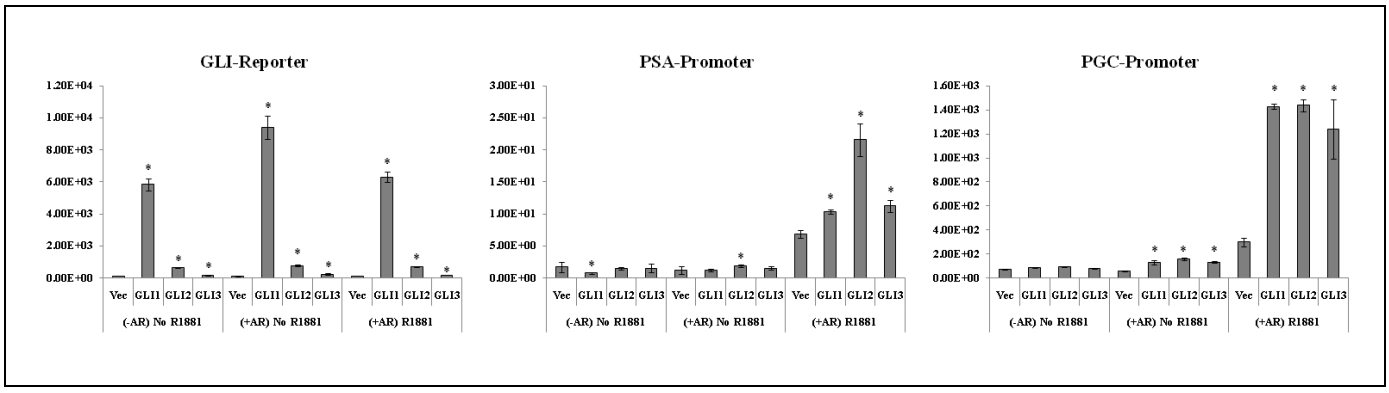

Figure 5. Co-expression of GLIs enhances AR transcriptional activity in FT293 cells. Luciferase-based promoter constructs (pGL4-GLI, pGL4-PGC, pGL3-PSA) with CMV-GFP plasmid was co-transfected with AR and GLI expressing plasmids into FT293 cells as indicated. Twenty-four hours after transfection, cells were switched to CSFBS media with or without 1nM R1881 and cultured for 24 hours. Cell extracts were quantified for luciferase activity that was normalized by GFP intensity. *: $p<0.01$ between GLI group and vector (Vec) control group.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that GLI1 expression is upregulated by androgen in prostate cancer cells with ETS gene fusion.
- Determination that GLI1 expression is not regulated by ETV1 in LNCaP cells.
- Generation of Lentiviral expression vectors to overexpress ERG, ETV1, and ETV4 in prostate cells.
- Generation of Tetracycline-inducible GLI1- and GLI2- overexpressing LNCaP cells for gene expression profiling.
- Identification of GLIs interacting with AR and enhancing AR transcriptional activity in prostate cells.

REPORTABLE OUTCOMES:

Published Manuscripts:

1. Chen M, Feuerstein MA, Levina E, et al. Hedgehog/Gli supports androgen signaling in androgen deprived and androgen independent prostate cancer cells. *Mol Cancer* 2010; 9: 89.
2. Chen M, Carkner R, Buttyan R. The Hedgehog/Gli Signaling Paradigm in Prostate Cancer. *Expert Rev of Endocrin Met,* 2011, In press

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CONCLUSION: Work in this project so far has suggested that ETS gene overexpression in prostate cancer can mimic the activity of the classical hedgehog signaling pathway through the ability of ETS transcription factors to directly upregulate expression of Gli genes in prostate cells. Activated GLIs are able to enhance androgen signaling in prostate cancer cells through interacting with AR.

REFERENCES:

1. Hu Y, Dobi A, Sreenath T, et al. Delineation of TMPRSS2-ERG splice variants in prostate cancer. *Clin Cancer Res* 2008; 14: 4719-25.
2. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. *Nat Rev Cancer* 2008; 8: 497-511.
3. Rickman DS, Pflueger D, Moss B, et al. SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. *Cancer Res* 2009; 69: 2734-8.
4. Tomlins SA, Laxman B, Dhanasekaran SM, et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* 2007; 448: 595-9.

5. Jiang J, Hui CC. Hedgehog signaling in development and cancer. *Dev Cell* 2008; 15: 801-12.
6. Wilson CW, Chuang PT. Mechanism and evolution of cytosolic Hedgehog signal transduction. *Development* 2010; 137: 2079-94.
7. Chen M, Feuerstein MA, Levina E, et al. Hedgehog/Gli supports androgen signaling in androgen deprived and androgen independent prostate cancer cells. *Mol Cancer* 2010; 9: 89.